Digestion of RNA of Chromatin and Nuclear Ribonucleoprotein by Staphylococcal Nuclease†

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ABSTRACT: Following a 1-h [^{3}H]uridine pulse of cells of a human colon carcinoma line, 15% of the radioactivity in heterogeneous nuclear RNA associated with both chromatin and nuclear ribonucleoprotein was not digested to acid soluble fragments during a 2-h incubation with staphylococcal nuclease (EC 3.1.4.7). These [^{3}H]uridine-labeled oligonucleotides were approximately 26 nucleotides in length. An RNA containing structure which sedimented no faster than 2 S could be isolated from the digests. Major and minor peptide species, of molecular weights 40 000 and 66 000, respectively, were associated with this structure isolated from either chromatin or nuclear ribonucleoprotein. The results demonstrate that some protein of nuclear ribonucleoprotein is complexed with the transcript while it is still associated with chromatin.

Rapidly labeled nuclear RNA of heterogeneous molecular weight (HnRNA)[1] can be isolated in the form of nuclear ribonucleoprotein (nRNP) free of DNA and chromatin protein (Georgiev and Samarina, 1971; Niessing and Sekeris, 1971; Pederson, 1974; Augenlicht and Lipkin, 1976). However, it is clear that, at least during synthesis, HnRNA must be intimately associated with chromatin, and several groups have recently reported that a considerable proportion of HnRNA is found in the chromatin fraction (Monahan and Hall, 1975; Tata and Baker, 1975; Augenlicht and Lipkin, 1976). We (Augenlicht and Lipkin, 1976) and Monahan and Hall (1975) have presented evidence that some of the chromatin HnRNA is precursor to HnRNA found free of chromatin in the form of nRNP. Tata and Baker, using a quite different technique, have concluded that in fact most poly(A)-rich HnRNA is located near its site of synthesis and in interchromatin granules within the nucleus. Fan and Penman (1971) have demonstrated that, during metaphase arrest, ribosomal precursor RNA is not processed and remains associated with chromatin.

It has been suggested that some of the RNA in nRNP is projected from nuclease digestion (Georgiev and Samarina, 1971; Stévenin and Jacob, 1974; Sekeris and Niessing, 1975; Faiferman and Pogo, 1975). We report here studies on the susceptibility of the RNA in both chromatin and nRNP to digestion by the nuclease from *Staphylococcus aureus* (EC 3.1.4.7). This enzyme was chosen because in the presence of Ca^{2+} it is a nonspecific nuclease (Cuatrecasas et al., 1967) and only nucleic acid sequences which are protected by protein will not be digested.

Materials and Methods

(*a*) *Cell Culture.* HT-29 human colon carcinoma cells, established by Dr. Jorgen Fogh of the Sloan-Kettering Institute, were grown in minimum Eagle's medium containing 15% fetal calf serum, 0.1 unit of penicillin/ml, 1 μg of streptomycin/ml, and 20 mM Hepes buffer as previously described (Augenlicht and Lipkin, 1976). These cells were used 4–6 days after plating and were in log phase.

(*b*) *Isotope Incorporation.* For incorporation of [^{14}C]thymidine into DNA, 0.01 μCi of [^{14}C]thymidine (49.7 mCi/mmol, New England Nuclear) was added per ml of culture medium on the day the cells were plated. For labeling of RNA, the cells were pulsed with 2 to 10 μCi of [^{3}H]uridine (44.5 Ci/mmol, Amersham/Searle) or 2.5 μCi of [^{14}C]uridine (500 mCi/mmol, Amersham/Searle) per ml of medium.

(*c*) *Cell Fractionation.* We have adapted the method of Bhorjee and Pederson (1973) to the isolation of chromatin and nRNP from these HT-29 cells. We have recently described this method in great detail elsewhere (Augenlicht and Lipkin, 1976). The chromatin obtained had a spectrum similar to that of other tissues (Bonner et al., 1968) and a protein–RNA–DNA ratio of 1.72:0.05:1. It should be pointed out that nucleoli are removed in this procedure, so very little ribosomal RNA should be present in the chromatin (Augenlicht and Lipkin, 1976). After fixation in formaldehyde by the method of Spirin et al. (1965), the buoyant density of the nRNP in $CsCl_2$ was 1.43, the nRNP therefore consisting of roughly 80% protein and 20% RNA. The nRNP sedimented at values up to 200 S (modal value of 76 S) on sucrose gradients. The RNA isolated from the nRNP had a mean sedimentation coefficient of 15 S as determined by sodium dodecyl sulfate–sucrose gradient centrifugation while that of chromatin was 20 S. In more recent experiments using the formamide gel system described below, the RNA isolated from nRNP was found to be quite heterogeneous, consisting of species of 4×10^3 to 10^6 molecular weight, while 48% of the RNA isolated from chromatin was at least 10^6 and the balance was of lower molecular weight.

Contamination of the nRNP by chromatin protein (or other extraneous protein) was not significant as suggested by the absence of histones in the nRNP and the fact that all of protein in nRNP banded at the same density (1.43) as the RNA in nRNP after formaldehyde fixation. In addition, consideration of the amount of DNA in the nRNP fraction led to an estimate that the maximum contamination of nRNP with chromatin protein was 5–10%. These arguments have been developed fully elsewhere (Augenlicht and Lipkin, 1976).

(*d*) *Digestion of Chromatin and nRNP.* This was essentially as described by Clark and Felsenfeld (1971). Chromatin and nRNP were exhaustively dialyzed against 5 mM sodium phosphate, pH 6.8, containing 25 μM $CaCl_2$. Aliquots were digested at 37 °C by addition of 5 μg of staphylococcal nu-

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[1] Abbreviations used: HnRNA, heterogeneous nuclear RNA; nRNP, nuclear ribonucleoprotein; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; uv, ultraviolet; UMP, uridine monophosphate.

clease (EC, 3.1.4.7; 10 000 units/mg, Worthington) in 0.1 ml of the same buffer. Final incubation volume was 1 ml. The reaction was stopped by the addition of 2 ml of cold 10% trichloroacetic acid. Calf thymus DNA (200 μg; Sigma) was added and the acid-precipitable material pelleted by centrifugation at 2000*g* for 10 min at 4 °C. A sample of the acid-soluble material was counted to determine the percent of nucleic acid digested.

(*e*) *Isolation of RNA.* RNA was isolated from whole cells, chromatin, and nRNP by the high-salt–DNase digestion procedure described by Soeiro and Darnell (1969). Nucleic acid was isolated from digested chromatin and nRNP by bringing the incubation mixture to 12 mM EDTA, extracting twice with buffer saturated phenol at room temperature, and precipitating the nucleic acid overnight with 2 volumes of 95% ethanol at −20 °C. Recovery of uridine pulse-labeled RNA was 60 to 70%.

(*f*) *Gel Electrophoresis.* Formamide gels of 5% were prepared and run according to the technique of Maniatis et al. (1975). Ten-centimeter cylindrical gels were run at 100 V/gel until the bromophenol blue tracking dye migrated to the end of the gel. For analysis of the oligonucleotides not digested by staphylococcal nuclease (Figure 2), the gels were run until the dye migrated 7.7 cm. The gels were resolved into 2-mm fractions with a Gilson gel mincer and counted in 10 ml of Aquasol (New England Nuclear) plus 5% distilled H_2O. Ten-centimeter, 10% sodium dodecyl sulfate–polyacrylamide gels were run and stained according to Weber and Osborn (1969).

(*g*) *Sucrose Gradients.* In order to analyze by sucrose gradient centrifugation (Figure 3) both the DNA and RNA containing structures which were not digested by staphylococcal nuclease, it was necessary to stop the digestion just as a visible precipitate formed during chromatin digestion (Clark and Felsenfeld, 1971, 1974). This was done by bringing the incubation mixtures to 5 mM EDTA. The samples were then dialyzed against two changes of 100 volumes of 5 mM sodium phosphate, pH 7.4, containing 0.2 mM EDTA, layered on linear 5–20% sucrose gradients (in the same buffer), and centrifuged as indicated in the text.

(*h*) *Analytical Ultracentrifugation.* The digested samples were prepared as for sucrose gradients (above), but dialyzed against sodium phosphate buffer containing 4% formaldehyde and finally against 1% formaldehyde, 30 mM NaCl. Analyses were carried out in a Beckman Model E analytical ultracentrifuge equipped with uv-photoelectric scanner optics in 12-mm double-sector cells at 48 000 rpm at 20 °C.

(*i*) *Isolation of a Structure Containing the Protected RNA.* In these experiments, advantage was taken of the fact that all of the protected DNA (complexed with histone) precipitates at the limit digest (Clark and Felsenfeld, 1971, 1974). Chromatin and nRNP were incubated for 2 h with staphylococcal nuclease and the digestion was then stopped by bringing the samples to 12 mM EDTA. The precipitate which forms at the completion of the chromatin digestion was removed by centrifugation at 3000*g* for 5 min. The supernatants were then fractionated on a 13 × 1 cm Sephadex G-150 column (Pharmacia). Elution was with 5 mM $NaPO_4$, pH 7.4, + 0.2 mM EDTA. The void volume was 2 ml and 0.5-ml fractions were collected. The fractions containing the protected RNA structures were pooled and centrifuged on sucrose gradients as described above.

(*j*) *Scintillation Counting.* All samples were counted in 10 ml in Aquasol (New England Nuclear) plus 5% distilled H_2O in an Intertechnique Scintillation counter. Efficiency was monitored by channel ratio using an external standard. A minimum of 2000 counts was observed for every sample counted, including each fraction from all gels and gradients.

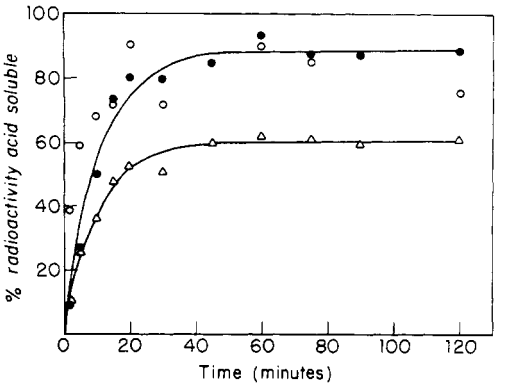

FIGURE 1: Digestion of nucleic acid in chromatin and nRNP by staphylococcal nuclease. Chromatin or nRNP was incubated with 5 μg of enzyme for the indicated time and the percent of total nucleic acid rendered acid soluble was determined. (△) [^{14}C]thymidine-labeled chromatin; (●) 1-h [^{3}H]uridine pulse labeled chromatin; (○) 1-h [^{3}H]uridine pulse labeled nRNP.

Results

Digestion of Nucleic Acid in Chromatin and nRNP. We have repeated the observation of Clark and Felsenfeld (1971, 1974), and found that no more than 60% of the [^{14}C]thymidine-labeled DNA in chromatin was rendered acid soluble during a 2-h incubation with staphylococcal nuclease (Figure 1). The digestion was complete by 1 h. Clark and Felsenfeld (1974) have shown that increasing the amount of enzyme does not affect the final extent of digestion, but only decreases the time necessary to reach the limit. Figure 1 also shows that only 80–90% of the radioactivity in chromatin, following a 1-h pulse of [^{3}H]uridine, was digested under the same conditions. Similarly, no more than 90% of the radioactivity in nRNP was digested (Figure 1).

These experiments were repeated in a number of separate experiments. In two such experiments, chromatin and nRNP were prepared from cells labeled with only [^{3}H]uridine for 1 h. Aliquots of each chromatin and nRNP preparation were digested in triplicate for 2 h. For the two independent chromatin preparations, averages of 86% (87, 84, 86) and 85% (88, 84, 82) of the [^{3}H]uridine incorporated into RNA were rendered acid soluble, while for the two nRNP preparations, the values were 81% (78, 79, 87) and 85% (84, 90, 81). Therefore, a maximum of about 85% of the radioactivity in both chromatin and nRNP following a 1-h pulse with [^{3}H]uridine was susceptible to digestion by staphylococcal nuclease.

Protein-free RNA isolated from undigested chromatin and nRNP was found to be 100% digested after a 2-h incubation. The protection from digestion was therefore due to the protein in these fractions. Moreover, the isolated RNA was 100% digested even in the presence of unlabeled chromatin or nRNP in the incubation mixture. It is therefore unlikely that the protection results from a gross redistribution of protein on nucleic acid as the digestion progresses. This point will be returned to in the Discussion.

Size of the Protected RNA. The size of the protected RNA was determined by formamide gel electrophoresis. Chromatin from cells pulsed for 1 h with [^{3}H]uridine was mixed with nRNP from cells similarly pulsed with [^{14}C]uridine. After a 2-h digestion, the nucleic acid was isolated. Figure 2 illustrates that the protected uridine labeled RNA of both chromatin and nRNP is the same size. It should be noted that the double label

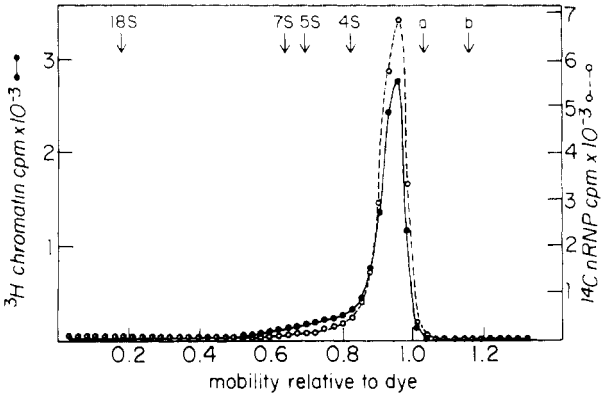

FIGURE 2: Formamide gel coelectrophoresis of RNA from digests of chromatin and nRNP. Cells were pulse labeled with either [^{14}C]- or [^{3}H]uridine for 1 h. The ^{3}H-labeled chromatin was then mixed with the ^{14}C-labeled nRNP, digested for 2 h, and the RNA extracted and analyzed on formamide gels. (●—●) [^{3}H]Chromatin RNA; (○- - -○) [^{14}C]nRNP RNA. The arrows indicate the position of markers run on parallel gels. The mobilities of 18S, 7S, and 5S rRNA and 4S tRNA were determined by running RNA extracted from whole cells, or rat liver ribosomes, and locating the positions of the main bands stained by methylene blue. Also run on the same gels were T_1 ribonuclease fragments of ^{32}P-labeled RNA which were located by autoradiography: (marker a) 17-base T_1 product No. 53-56 of 5S RNA of KB cells (Forget and Weissman, 1968); (marker b) 9-base T_1 product No. 61 of VA RNA (Ohe and Weissman, 1971). The relative mobility is the number of centimeters migrated/centimeter bromophenol blue migrated. A plot of the log molecular weight vs. the relative mobility was linear for the six standards shown.

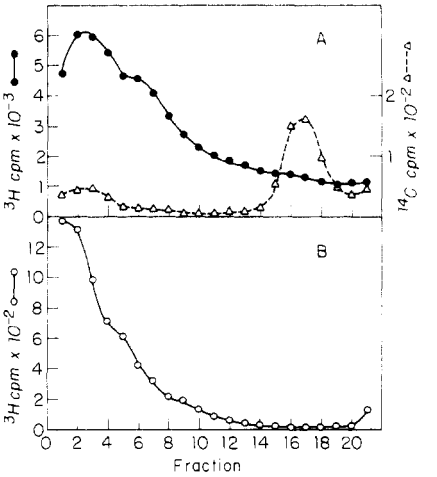

FIGURE 3: Sucrose gradient analysis of chromatin and nRNP digests. The fractions were incubated with staphylococcal nuclease until a precipitate was first seen in the chromatin fraction (in this experiment, 1 h). The digests were then layered on 5–20% sucrose gradients in 5 mM $NaPO_4$ buffer, pH 7.4, + 0.2 mM EDTA and centrifuged at 37 000 rpm in the Beckman SW41 rotor at 4 °C for 21 h. (A) Chromatin digest: (△- - -△) [^{14}C]thymidine labeled; (●—●) [^{3}H]uridine, 1-h pulse labeled. (B) nRNP digest: (○—○) [^{3}H]uridine, 1-h pulse labeled.

experiment eliminates the problem of variability in digestion, extraction, or between gels. We estimate that the protected RNA is 26 nucleotides in length (see legend to Figure 2). Similar results were obtained when the labels were reversed, and in single label experiments.

Analysis of the Structure Containing the Protected RNA. The digested chromatin and nRNP were examined by sucrose gradient centrifugation. The digestion was stopped when the first visible precipitate was seen (Methods). It is clear that, in chromatin, the [^{3}H]uridine-labeled protected RNA did not cosediment with the structure containing the protected DNA (Figure 3A). The DNA-containing structure had a sedimentation coefficient of 12.1 S as determined by analytical ultracentrifugation.

Most of the radioactivity in RNA of chromatin and nRNP was at the top of the gradients shown in Figures 3A and 3B. These are principally oligonucleotides (note the residual amount of digested DNA fragments which were not lost upon dialysis and also sediment at the top of the gradient). However, there is a shoulder of faster sedimenting radioactivity in RNA in the digested chromatin fraction, which is also present, though less distinct, in the digested nRNP. This corresponds to a minor component seen in the analytical runs of the digested chromatin and nRNP. In both cases, this component sedimented no faster than 2 S, although an accurate value was not obtained due to the small amounts of material and low sedimentation rate.

Isolation of a Structure Containing the Protected RNA. Following a 2-h digestion of the chromatin, all of the protected DNA–protein complex precipitated and was removed by low-speed centrifugation (see Materials and Methods). This also pelleted 16% of the [^{3}H]uridine-labeled RNA. However, only 58% of this pelleted RNA was acid precipitable and 9% of the nonpelleted RNA was acid precipitable. Therefore, of the 15% of the [^{3}H]uridine-pulse-labeled RNA in chromatin protected from digestion, about one-half was lost when the protected DNA precipitate was pelleted. The loss of acid-soluble and insoluble material at this step most likely represents nonspecific trapping by the large DNA–histone precipitate. In fact, most of the radioactivity in this pellet (92%) could be resolubilized by simply washing the pellet with 5 mM phosphate buffer–0.2 mM EDTA, pH 7.4.

The elution patterns of the digested chromatin (supernatant) and nRNP from Sephadex G-150 columns are shown in Figures 4A and 4B, respectively. In both fractions, there was a small peak of radioactivity which eluted just with the void volume. This was followed by the bulk of the digested labeled RNA, which was all acid soluble. In chromatin (Figure 4A) the digested DNA was also eluted in the second major peak (all of the protected DNA had precipitated and been removed). The fractions containing the first small peak were pooled as indicated (Figure 4A,B) and further analyzed on sucrose gradients as in Figure 3. The isolated structure from both chromatin and nRNP containing the [^{3}H]uridine-pulse-labeled, protected RNA fragment sedimented uniformly in the same position as the shoulders seen when the whole digests were similarly centrifuged (Figure 3). The material from each of these sucrose gradients was pooled and concentrated, and the protein was analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 5).

For both chromatin and nRNP, a main band was seen corresponding to a molecular weight of approximately 40 000. The minor band with a molecular weight of 66 000 is a doublet. The other minor bands were variable, probably due to the small amounts of material available in several experiments. The 40 000 and 66 000 molecular weight bands were also the only proteins observed when material from the RNA shoulder of the total digests (Figure 3A,B) was analyzed on similar gels. Gels of chromatin, nRNP, and staphylococcal nuclease are shown for comparison.

To demonstrate that the protein recovered from the gradients was associated with RNA, the following experiment was done. Preparations of nRNP and chromatin were digested for 2 h with staphylococcal nuclease as described. Five micrograms of pancreatic A and 12.5 units of T_1 ribonuclease were added to the incubation mixture for the last hour of digestion. These

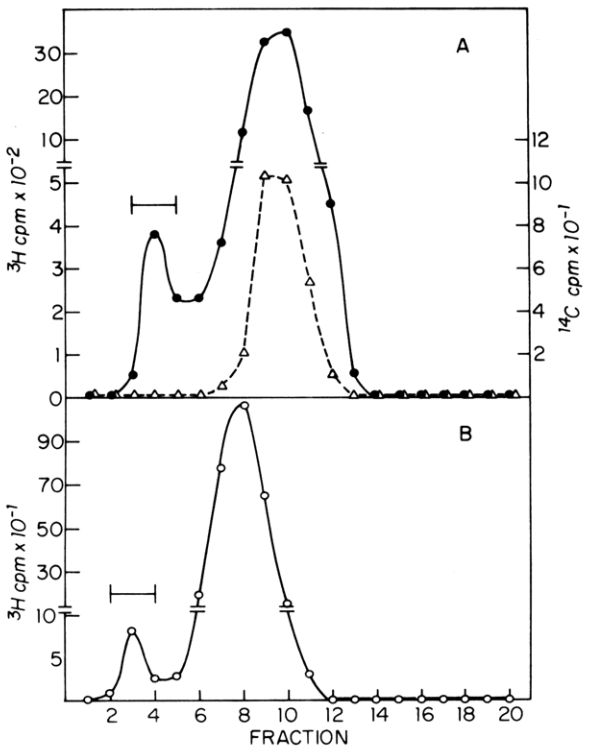

FIGURE 4: Sephadex G-150 chromatography of digests of chromatin and nRNP. Following a 2-h incubation with 5 μg of staphylococcal nuclease, the fractions were centrifuged at 3000 g for 5 min to remove precipitated protein and nucleic acid. The supernatants were then fractionated on 13 × 1 cm columns of Sephadex G-150, equilibrated and run with 5 mM $NaPO_4$, pH 7.4 + 0.2 mM EDTA. (A) Chromatin: (Δ- - -Δ) [^{14}C]thymidine labeled; (●—●) 1-h [^{3}H]uridine pulse labeled. (B) nRNP: (○—○) 1-h [^{3}H]uridine pulse labeled.

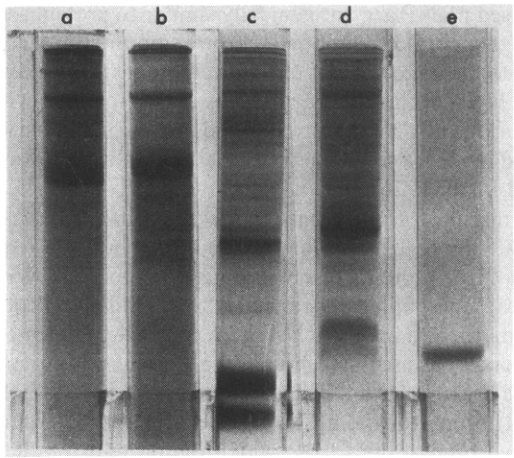

FIGURE 5: Sodium dodecyl sulfate gel electrophoresis. The fractions from the sucrose gradients (see text) were pooled, concentrated, and analyzed by sodium dodecyl sulfate gel electrophoresis: (a) digested chromatin; (b) digested nRNP; (c) whole chromatin; (d) whole nRNP; (e) staphylococcal nuclease.

enzymes render >99% of the 1-h [^{3}H]uridine-pulse-labeled RNA of nRNP and chromatin acid soluble (Kish and Pederson, 1975; and data not shown). The isolation procedure was again carried out. The final gradients were divided into five fractions, and each fraction was concentrated and analyzed by sodium dodecyl sulfate gel electrophoresis. In no case did we find any protein bands when the chromatin and nRNP had been terminally digested with pancreatic A and T_1 ribonuclease. This indicates that the bands seen on the gels of Figure 4 (A and B) were due to protein associated with the oligonucleotides.

Discussion

We have shown that 15% of the radioactivity in HnRNA associated with both chromatin and nRNP following a 1-h uridine pulse is not digested to acid-soluble fragments during a 2-h incubation with staphylococcal nuclease. For both chromatin and nRNP, these [^{3}H]uridine-labeled oligonucleotides are approximately 26 nucleotides in length. An RNA-containing structure can be isolated from the digests which sediments no faster than 2 S and is associated with major and minor protein species of 40 000 and 66 000 daltons, respectively, and other minor species. It is presumably these proteins which protect the RNA from digestion since isolated RNA is completely digested under the same conditions. Protection from nuclease by protein of a portion of nuclear RNA has been noted for nRNP in fixed tissue (Swift, 1959; Monneron and Bernhard, 1969; Snow and Callan, 1969) and also in isolated nRNP (Georgiev and Samarina, 1971; Stévenin and Jacob, 1974; Sekeris and Niessing, 1975; Faiferman and Pogo, 1975). We do not as yet know whether the protected piece is a specific sequence, if it is present in each HnRNA molecule, or the number of such regions per molecule. We have not been able to isolate protein–RNA complexes of high molecular weight following partial staphylococcal nuclease digestion of the nRNP (40–70%). This is most likely due to the early action of the enzyme as an endonuclease followed by exonuclease activity (Alexander et al., 1961).

The presence of HnRNA in the chromatin fraction is not surprising since this is its site of synthesis, and is consistent with the observations that the RNA:DNA ratio is highest in puffs (Edström and Beermann, 1962), lampbrush chromosomes (Izawa et al., 1963), and in the chromatin from tissues with the highest capacities for RNA synthesis (Dingman and Sporn, 1964; Hjelm and Huang, 1975). Several groups have recently reported that chromatin can be isolated with HnRNA still associated and that some of this chromatin RNA is precursor to RNA found free of chromatin (Daneholt, 1972; Monahan and Hall, 1975; Tata and Baker, 1975; Augenlicht and Lipkin, 1976), although some of the RNA may also be stored in chromatin (Berendes, 1968; Sommerville, 1973). Interestingly, Fan and Penman (1971) have shown that, during mitotic arrest, ribosomal RNA is not processed and remains associated with chromatin. It is not clear to what extent processing takes place during association of the RNA with chromatin (Tata and Baker, 1975).

The presence of this RNA as RNP associated with chromatin regions active in transcription is well-documented morphologically in several systems. These include the lampbrush chromosomes of amphibian oocytes (Gall and Callan, 1962; Snow and Callan, 1969; Miller and Hamkalo, 1972; Miller and Bakken, 1972), the puffs of insect polytene chromosomes (Beermann and Bahr, 1954; Swift, 1959; Stevens and Swift, 1966), and most recently in mammalian cells (Miller and Bakken, 1972). RNA in the form of RNP has also been clearly demonstrated in the nucleus free of chromatin (Beermann and Bahr, 1954; Swift, 1959; Stevens and Swift, 1966; Monneron and Bernhard, 1969).

Since protein is therefore associated with the transcript both during its association with chromatin and after its release, it is not surprising that we have found that the protection from digestion of both classes of RNA is similar, and apparently due in both cases to similar proteins. This is consistent with the report of Scott and Sommerville (1974) that the proteins of

released nRNP are antigenically similar to protein associated with the loops of lampbrush chromosomes. However, in contrast to the large amount of relatively stable protein complexed with RNA while the transcript is still associated with chromatin, rapidly turning over minor protein species were not found in nRNP until the transcript was released (Augenlicht and Lipkin, 1976).

It might be argued that the protection of a small region of RNA from digestion could be brought about by a rearrangement of the protein present as digestion proceeds. We believe several lines of evidence make this unlikely. Protein-free chromatin and nRNP RNA were completely digested under the conditions used here, even if the digestions were carried out in the presence of unlabeled chromatin and nRNP (the unlabeled chromatin and nRNP were equivalent in amount to the labeled preparations from which the protein-free RNA had been prepared). This indicates that there is no gross redistribution of protein during digestion under these conditions, a conclusion that was also reached by Felsenfeld based on more extensive experiments of chromatin digestion under identical conditions (Clark and Felsenfeld, 1971, 1974; Sollner-Webb and Felsenfeld, 1975). This type of experiment does not, however, eliminate the possibility that more subtle protein rearrangement, such as sliding along the polynucleotide, might contribute to the protection. Although initial results indicate that the extent of protection is similar in chromatin and nRNP which were fixed with formaldehyde by the method of Spirin et al. (1965), this experiment is again not conclusive since Doenecke and McCarthy (1975) have demonstrated that most of the nonhistone protein in chromatin cannot be fixed to nucleic acid. The best evidence that the structure we isolate containing the protected RNA fragment is not due to random association with protein comes from analysis of the structure itself. First, we have shown that the proteins associated with the protected RNA are of specific molecular weights (Figure 5). Second, base analysis indicates that the protected RNA fragments have a very high G + C content (60–75%) and mapping of oligonucleotides produced by T_1 ribonuclease shows that the fragments have many GG sequences.[2] This suggests that the sequences protected are not random. Finally, we should point out that, while the RNA is protected from digestion by staphylococcal nuclease, it is not protected from either T_1 or pancreatic A ribonuclease, again indicating that the protection is not due to simple aggregation or precipitation.

Kish and Pederson (1975) have recently reported that >99% of the uridine-pulse-labeled HnRNA in nRNP is rendered acid soluble by a 75-min digestion with pancreatic A and T_1 ribonuclease. We have made this same observation in our experiments under the same conditions we used for staphylococcal nuclease digestion. It is, therefore, clear that the results with staphylococcal nuclease, on the one hand, and pancreatic A + T_1 ribonuclease, on the other, are not comparable. In addition, the use of staphylococcal nuclease allowed us to remove chromatin proteins by precipitation of the limit digest and therefore enabled us to also study protein of chromatin associated HnRNA.

The undigested fragment studied by Kish and Pederson (1975) is predominantly the poly(A) region but also contains 17.7 mol % UMP. This fragment is associated with a major polypeptide species of 74 000 molecular weight and a minor species of 86 000 and these are equated with the poly(A)-associated proteins first reported by Kwan and Brawerman (1972) and Blobel (1973). It is not clear whether the major and minor species reported here are homologous to the polypeptides reported by these authors, but the differences in molecular weight and the association in our experiments with about 15% of the uridine pulse labeled RNA make this unlikely.

Alternatively, the 40 000 molecular weight major species could be analogous to the peptide of the same size which has been reported to be the only protein associated with HnRNA to form informofers (Georgiev and Samarina, 1971; Martin, et al., 1974). However, in agreement with several other authors, we find a wide sprectrum of peptide species in our nRNP preparation (Niessing and Sekeris, 1971; Ducamp and Jeanteur, 1973; Pederson, 1974; Gallinaro-Matringe et al., 1975; Augenlicht and Lipkin, 1976). In fact, in our system, the major peptide species of total nRNP has a molecular weight of 34 000 rather than 40 000 and therefore runs in a different position on sodium dodecyl sulfate gels (Figure 5).

Acknowledgments

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Effects of α-Amanitin, Cycloheximide, and Thioacetamide on Low Molecular Weight Nuclear RNA†

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ABSTRACT: Studies were made on the effects of α-amanitin, cycloheximide, and thioacetamide on synthesis and content of low molecular weight nuclear RNA. Cycloheximide, an inhibitor of protein synthesis and the synthesis of 45S pre-rRNA and 5S RNA, also inhibited synthesis of nuclear U1 and U3 RNAs. α-Amanitin, an inhibitor of DNA-dependent RNA polymerase II, inhibited the synthesis of U1 and U2 low molecular weight nuclear RNA. Thioacetamide, which induces nucleolar hypertrophy and increased nucleolar RNA polymerase activity, markedly increased synthesis of 5.8S RNA and U3 RNA. These results show that syntheses of individual low molecular weight nuclear (LMWN) RNAs are controlled by different regulatory mechanisms. In particular, there appears to be a specific relationship between U3 RNA and functional states of the nucleolus.

Of the low molecular weight nuclear RNAs with sedimentation values of 4 to 8 S, the complete nucleotide sequences of 4.5S RNA_I, U1 RNA, and U2 RNA have been defined (Ro-Choi et al., 1972; Reddy et al., 1974; Shibata et al., 1974, 1975). U1 and U2 RNA have the common feature of a highly methylated 5′ terminus which contains the unusual nucleotide $pm_3^{2,2,7}G$ in a pyrophosphate linkage to the 5′ portion of the molecule (Ro-Choi et al., 1974, 1975) and they exist in the nuclei as ribonucleoprotein complexes (Raj et al., 1975). The 4.5S, U1, and U2 RNA are extranucleolar in location; U3 RNA is nucleolus specific (Busch et al., 1971; Ro-Choi and Busch, 1974). These RNA species have been found in tissues of several vertebrate species (Moriyama et al., 1969; Hodnett and Busch, 1968; Weinberg and Penman, 1968; Dingman and Peacock, 1968; Rein and Penman, 1969; Zapisek et al., 1969). U1, U2, and U3 RNA are metabolically very stable and do not appear to be precursors to cytoplasmic products.

The use of inhibitors which have differential effects on different RNA polymerases or interfere with nucleic acid processing should aid in elucidating the functions of these LMWN RNAs. α-Amanitin is a specific inhibitor of nucleoplasmic RNA polymerase II in vitro (Stirpe and Fiume, 1967) but when administered to rats, α-amanitin inhibits both nucleoplasmic and nucleolar RNA synthesis (Jacob et al., 1970;

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